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Regulation of expression of the lung amiloride-sensitive Na^+ channel by steroid hormones

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Molecular cloning of the amiloride-sensitive Na^+ channel has permitted analysis of the mechanisms of its stimulation by steroids. In rat lung cells in primary culture, where its mRNA has been detected, the activity of an amiloride-sensitive channel, highly selective for Na^+, is controlled by corticosteroids. Dexamethasone (0.1 μM) or aldosterone (1 μM) induced, after a minimum 10 h treatment, a large increase of the amiloride-induced hyperpolarization and of the amiloride-sensitive current. A parallel increase in the amount of the mRNA was observed. The corresponding gene is thus a target for steroid action. Using synthetic specific agonists and antagonists for mineralo- and glucocorticoid receptors, it has been shown that the steroid action on Na^+ channel expression is mediated via glucocorticoid receptors. Triiodothyronine, known to modulate steroid action in several tissues, had no effect on both the amiloride-sensitive Na^+ current and the level of the mRNA for the Na^+ channel protein, but potentiates the stimulatory effect of dexamethasone. The increase in Na^+ channel activity observed in the lung around birth can thus be explained by a direct increase in transcription of the Na^+ channel gene.

Key words: amiloride/lung/Na^+ channel/steroids/triiodothyronine

Introduction

An active transcellular sodium reabsorption, linked to a K^+ transcellular secretion, occurs in numerous epithelia, such as renal cortical collecting tubule, distal colon and reabsorptive duct of sweat glands (Garty, 1986). Mineralocorticoid hormones enhance Na^+ reabsorption as well as K^+ secretion, and play a major role in extracellular salt homeostasis (Morel and Doucet, 1986). The apical amiloride-sensitive Na^+ channel (Crabbé, 1961), the baso-lateral Na^+/K^+-ATPase (Jørgensen, 1969; Rossier and Palmer, 1992) and baso-lateral K^+ channels (Tsuchiya *et al.*, 1992; Urbach and Harvey, 1993) are the key elements of the ion transport machinery. They are regulated by aldosterone, either by stimulation of their activity and/or by augmentation of their synthesis (reviewed by Rossier and Palmer, 1992).

In pulmonary epithelium, the reabsorption of fluid from the alveolar space to the blood is also associated with active Na^+ transport (Mason *et al.*, 1982; Goodman *et al.*, 1983; Basset *et al.*, 1987). As for the other tight epithelia, Na^+ transport across the alveolar epithelium is carried out through apical amiloride-sensitive Na^+ channels (Goodman *et al.*, 1983, 1987; Basset *et al.*, 1987; Cheek *et al.*, 1989; Russo *et al.*, 1992). Recently, a highly Na^+-selective and highly amiloride-sensitive 4 pS Na^+ channel has been characterized in rat lung epithelial cells (Voilley *et al.*, 1994). It is very similar to channels previously described in rat cortical collecting tubule (Palmer and Frindt, 1986), A6 cells (Hamilton and Eaton, 1986), chicken coprodeum (Clauss and Skadhauge, 1988) or toad urinary bladder (Frings *et al.*, 1988). Its molecular structure is identical to that of the Na^+ channel from rat colon (Canessa *et al.*, 1993; Lingueglia *et al.*, 1993), as demonstrated by the molecular cloning of the human lung Na^+ channel (Voilley *et al.*, 1994).

Glucocorticoid (Ballard *et al.*, 1974; Giannopaulos, 1974) and mineralocorticoid (Krozowski and Funder, 1981) receptors are present in both fetal and adult lung, and as the other tight epithelia, the lung epithelium is a target for steroid regulation: (i) aldosterone is known to regulate an apical Na^+ conductance in *Xenopus laevis* lung epithelium (Fischer and Clauss, 1990), and in canine tracheal epithelial cells in culture (Cullen and Welsh, 1987); (ii) corticosteroids are known to play a role in the maturation of fetal lung before birth in relation to its ability to reabsorb Na^+ (O'Brodovich, 1991; Bland and Nielson, 1992). Moreover, around birth, the ionic transport in the lung switches from a Cl^--secreting to a Na^+-reabsorbing tissue, together with a parallel increase in the Na^+ channel transcription; this results in the clearance of the pulmonary fluid as the lung switches to an air-conducting system (Voilley *et al.*, 1994).

In the present work, lung epithelial cells in culture have been used as a model in order to understand the molecular mechanisms governing the activation of the Na^+ channel by steroids. Molecular properties of the Na^+ channel regulation have been studied by a combination of electrophysiological and molecular biology techniques, after treatment by natural steroids, and specific agonists or antagonists of mineralocorticoid or glucocorticoid receptors (Philibert *et al.*, 1985).

Results and discussion

Patch-clamp and biochemical experiments were conducted on primary cultures of lung epithelial (LE) cells from 21-day-old fetal rat as previously described (Voilley *et al.*, 1994). Typical whole-cell recordings presented in Figure 1 show that a 24 h exposure of LE cells to 1 μM aldosterone or 0.1 μM dexamethasone induced: (i) a depolarization of the membrane (Figure 1a); (ii) a large increase in the amplitude of the amiloride-induced hyperpolarization component (AIH) (Figure 1a); and (iii) a large increase in the current changes

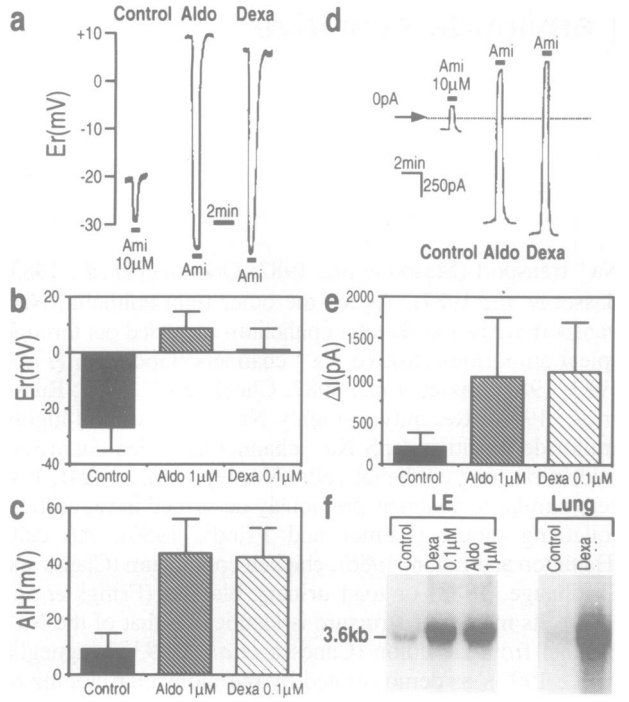

Fig. 1. (**a**) Typical recording showing the effect of 10 μM amiloride on the resting membrane potential in control conditions or after 24 h treatment with 1 μM aldosterone or 0.1 μM dexamethasone. (**b** and **c**) Histograms showing their effect on the mean resting membrane potential (b) and on the mean amiloride-induced hyperpolarization (AIH) (c). (**d**) Typical recording showing the effect of 10 μM amiloride on the amiloride-induced current changes in control conditions or after 24 h treatment with 1 μM aldosterone or 0.1 μM dexamethasone. (**e**) Histograms showing their effect on the mean amiloride-induced current change. Results are the mean ± SD of 15 measurements made on three different cultures. (**f**) Northern blot experiments with total RNA from LE cells treated or not with 0.1 μM dexamethasone or 1 μM aldosterone during 8 h (left), and total lung of adult rat infused three times (once every 12 h) with corn oil ± 3 mg dexamethasone (right).

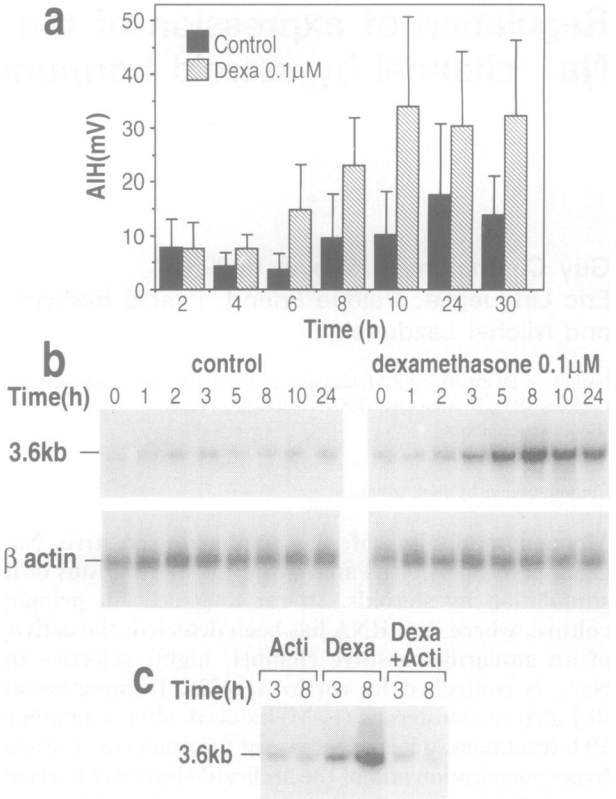

Fig. 2. (**a**) Histogram showing the mean AIH measured at various times in the absence or presence of 0.1 μM dexamethasone. Time $t = 0$ corresponds to the time at which treatment with dexamethasone began. Results are the mean ± SD of 45 measurements made in nine different cultures. (**b**) Northern blot studies of induction by ± 0.1 μM dexamethasone of the NaCh mRNA and control with β actin. (**c**) Northern blot studies after treatment of LE cells with 0.1 μM dexamethasone ± 5 μg/ml actinomycin D.

induced by amiloride (Figure 1d). Mean values calculated from experiments performed on five different cultures are also presented in Figure 1. They show a change in the resting potential of LE cells from −27 ± 9.4 mV in the control to +8.4 ± 6.8 mV and to +0.8 ± 7.8 mV after a 24 h exposure to aldosterone and dexamethasone, respectively (Figure 1b). The AIH increased from 9 ± 5.9 mV in control conditions to 43.7 ± 12.4 mV with aldosterone and to 42.4 ± 10.4 mV with dexamethasone (Figure 1c). The amiloride-induced current change increased from 211 ± 163 pA in the control to 1052 ± 699 pA with aldosterone and to 1102 ± 590 pA with dexamethasone (Figure 1e). In the following part of this paper, AIH is used as an index of changes in the amiloride-sensitive Na^+ conductance.

Northern blot analysis, using a fragment from the previously cloned rat colon Na^+ channel (Lingueglia *et al.*, 1993) as a probe, shows that both 1 μM aldosterone and 0.1 μM dexamethasone induced, after an 8 h exposure, a large increase in the amount of mRNA of the amiloride-sensitive Na^+ channel in LE cells (Figure 1f). The same kind of induction was observed using total lung of adult rats infused with dexamethasone (Figure 1f).

The time course of steroid action on epithelial Na^+ channel function is presented in Figure 2. Cultured LE cells placed in the presence of dexamethasone (0.1 μM) showed a latency period of 1−2 h, after which a continuous increase in the AIH could be observed up to 10 h (Figure 2a). A 3- to 4-fold increase in AIH was usually observed after a 10−15 h treatment. The time course of the induction by dexamethasone (0.1 μM) of the amiloride-sensitive Na^+ channel (NaCh) was also studied by the Northern blot technique. Figure 2b shows that the amount of amiloride-sensitive Na^+ channel mRNA reached a maximum increase after 8 h of treatment, with a lag period of 2 h. This increase in the mRNA level for NaCh is due to *de novo* synthesis since there was no increase in the mRNA for NaCh when dexamethasone was added together with actinomycin D (5 μg/ml) which inhibits transcription (Figure 2c).

The concentration dependence of the dexamethasone effect after a 15 h exposure is shown in Figure 3. An increase in AIH of ~10 mV could be observed with 0.1 nM dexamethasone. The maximum increase (~30 mV) was observed with 10 nM dexamethasone. Northern blot results are parallel to electrophysiological data (Figure 3). The same results were obtained with aldosterone. However, 10-fold higher concentrations of aldosterone were required to observe similar increases in AIH and in the level of NaCh mRNA.

Aldosterone is known to exert its action via two types of receptors (reviewed by Rossier and Palmer, 1992). The type

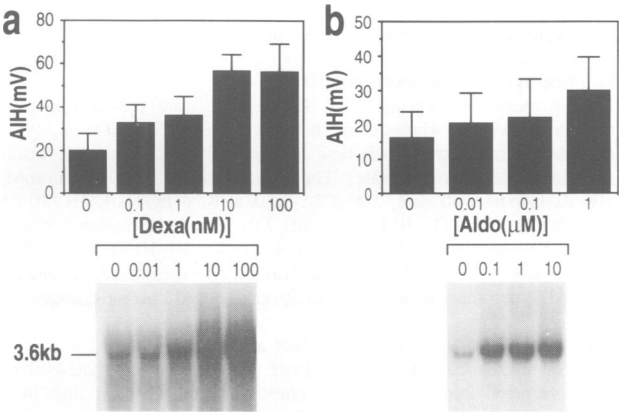

Fig. 3. Dose−response relationships for the effect of dexamethasone (**a**) and aldosterone (**b**). Measurements of AIH were made after 15−20 h exposure of LE cultures to the hormones. Number of experiments, $n = 30$ in (a); $n = 36$ in (b). Northern blot analysis was done after 8 h exposure to dexamethasone (a) and aldosterone (b).

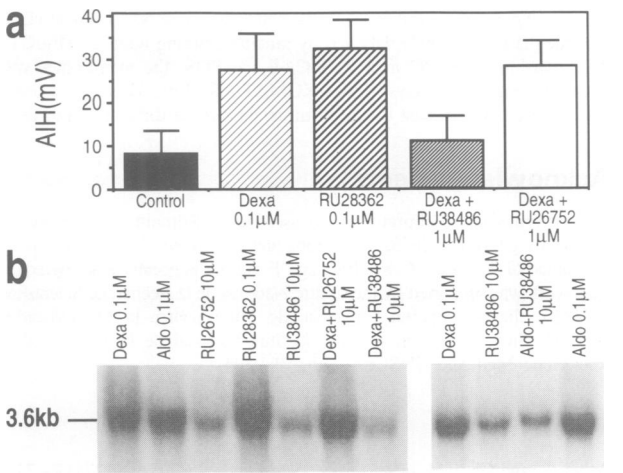

Fig. 4. (**a**) Histogram showing mean (AIH) values measured in control conditions after 15 h exposure of LE cells to dexamethasone, the glucocorticoid agonist RU 28362, 0.1 μM dexamethasone and the glucocorticoid antagonist RU 38486, and 0.1 μM dexamethasone and the mineralocorticoid antagonist RU 26752. Results are the mean ± SD of 15 measurements made on three different cultures. (**b**) Before Northern blot analysis, LE cells were treated for 8 h with 0.1 μM dexamethasone or glucocorticoid agonist RU 28362, with 0.1 μM dexamethasone + 10 μM mineralocorticoid agonist (RU 26752) or glucocorticoid agonist (RU 38486), and with 0.1 μM aldosterone.

I or mineralocorticoid receptor has a high affinity for aldosterone, while the type II or glucocorticoid receptor has a lower affinity for the hormone, but better affinities for synthetic glucocorticoids such as dexamethasone (Rossier and Palmer, 1992). The observation (Figures 1 and 3) that 0.1 μM dexamethasone and 1 μM aldosterone are nearly equally effective in increasing AIH suggests that the aldosterone effect is mediated mainly via the glucocorticoid receptor. However, although dexamethasone is known to have a high affinity for the glucocorticoid type II receptor, it also interacts with mineralocorticoid receptors (Rossier and Palmer, 1992). It was therefore necessary to use specific glucocorticoid or mineralocorticoid agonists and antagonists to determine the receptor type. Figure 4a shows that the stimulatory effects of dexamethasone (0.1 μM) on AIH (27.6 ± 8.3 mV) were also produced by the specific synthetic glucocorticoid agonist RU 28362 (0.1 μM) (32.4 ± 6.7 mV). Moreover, the stimulatory effects of dexamethasone were found to be inhibited by the specific glucocorticoid antagonist RU 38486 (1 μM) (AIH = 11.0 ± 6.0 mV), but not by the mineralocorticoid antagonist RU 26752 (1 μM) (AIH = 28.4 ± 5.8 mV). The same type of results were obtained at the molecular level using Northern blot experiments (Figure 4b). The conclusion is that dexamethasone exerts its action primarily via binding to glucocorticoid type II receptors in LE cells.

It has been previously demonstrated that the combined administration of glucocorticoid and thyroid hormones induces a Na^+-absorptive capacity in the immature fetal lung (Barker *et al.*, 1990). In rat cortical collecting duct, treatment of the tissue with triiodothyronine (T_3) is necessary for *in vitro* induction of Na^+/K^+-ATPase by aldosterone (Barlet-Bas *et al.*, 1988). For these reasons, we also investigated the effect of T_3 on the stimulatory action of dexamethasone on LE cells. Table I illustrates the results obtained with eight different cultures. Whereas T_3 alone did not affect AIH even after 10−15 h treatments [from data obtained on three different cultures, the mean AIH was 8.7 ± 9.2 mV ($n = 17$) in control conditions and 7.7 ± 8.9 mV ($n = 20$) in the presence of 0.1 μM T_3], a significant increase ($P < 0.05$) in the effect of dexamethasone on AIH by T_3 could be observed in four out of eight experiments. The same type of synergistic effect of dexamethasone and T_3 was also observed by Northern blot analysis (Figure 5). As glucocorticoids and T3 levels are known to increase *in vivo* around birth (Barker *et al.*, 1988, 1990), their inducing effect on the Na^+ absorptive capacity in immature fetal

Table I. Synergistic effect of T_3 and dexamethasone on amiloride-induced hyperpolarization

	Control	Dexamethasone (0.1 μM) + T_3 (0.1 μM)	Dexamethasone (0.1 μM)
1	2.8 ± 1.7	11.6 ± 4.0	22.6 ± 3.1*
2	21.0 ± 1.7	38.1 ± 8.2	48.2 ± 5.9*
3	20.4 ± 6.9	43.6 ± 6.8	52.0 ± 7.9*
4	6.0 ± 7.0	18.0 ± 5.5	26.3 ± 8.7*
5	14.2 ± 6.9	25.0 ± 5.3	20.1 ± 7.0
6	8.8 ± 6.1	24.6 ± 7.3	23.4 ± 6.3
7	14.4 ± 3.2	39.8 ± 10.9	30.0 ± 3.2
8	7.0 ± 4.3	17.2 ± 7.6	18.8 ± 5.1

Mean values (in mV) were calculated from at least five different cells for each experimental condition. Values are given as means ± SD.
*$P < 0.05$ using Student's unpaired *t*-test.

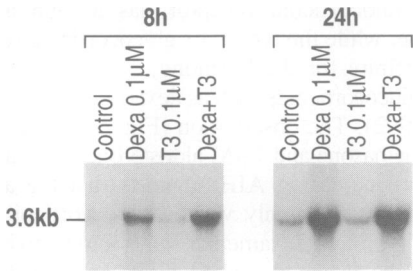

Fig. 5. Studies by Northern blot of the treatment with 0.1 μM dexamethasone in the presence or absence of 0.1 μM T_3.

lung can be explained by their action on Na^+ channel transcription.

Several mechanisms have been previously proposed to explain the effect of aldosterone on Na^+ channel expression in the so-called 'late' response Na^+ channel activity to steroid treatment. Neosynthesis has been thought to involve channel-forming or channel-regulatory proteins, presumably through an interaction with a type II receptor (Asher and Garty, 1988). However, recruitment of plasma membrane Na^+ channels from an internal pool of silent channels has also been suggested (Palmer *et al.*, 1982). This process could be produced either by changes in internal pH (Harvey and Ehrenfeld, 1988), or by methylation reactions (Sariban-Sohraby *et al.*, 1984) and/or via the phospholipid metabolism (Goodman *et al.*, 1971). The present paper documents the effect of steroids on the corresponding NaCh subunit in lung (Canessa *et al.*, 1993; Lingueglia *et al.*, 1993) via a binding to type II receptors and a control of its transcription. These findings might have a bearing on the facts that: (i) glucocorticoids have an important role in the development of the lung (O'Brodovich, 1991; Bland and Nielson, 1992); (ii) an abnormal increase in amiloride-sensitive Na^+ transport is observed in diseases such as cystic fibrosis (Boucher *et al.*, 1986); (iii) glucocorticoids are used in the treatment of asthma (Chrétien and Marsac, 1990) and other lung diseases (Ferrazzini *et al.*, 1987). Alteration of Na^+ channel activity might be involved in the side effects of these drugs in asthma and in their beneficial effects in acute mountain sickness, which is associated with the formation of peripheral edemas (Ferrazzini *et al.*, 1987).

Finally, this paper demonstrates the key role of the cloned RCNaCh protein in the control of Na^+ channel expression by steroids. This regulation, at least in the lung, occurs via an increase in the transcription of this channel protein, and is synergized by T_3.

Materials and methods

LE cell culture

Primary cultures of fetal alveolar type II cells were carried out according to Orser *et al.* (1991) with minor modifications. Briefly, the lungs from Wistar fetal rats of 18–21 days gestation were removed and minced in Hank's balanced salt solution (HBSS), washed to remove erythrocytes and stirred for 20 min at room temperature in the presence of 0.125% trypsin (Seromed) and 0.002% DNase type II (Boehringer). The cell suspension was discarded and the remaining pieces further dissociated in 0.06% collagenase (Worthington) until the solution was cloudy (15–25 min). The supernatant was centrifuged at 1000 r.p.m. for 5 min. The pellet resuspended in the growth medium was filtered through an 80 μm nylon mesh filter. The cells were incubated for 3 h in 95% air–5% CO_2 at 37°C to remove fibroblasts by differential adherence, resuspended in MEM D-valine, 0.1 mM non-essential amino acids (Sigma), 10% fetal calf serum (Boehringer) and seeded onto tissue culture dishes coated with collagen (Falcon). The cells were studied 24 h–8 days after plating.

Electrophysiological experiments

Resting membrane potential and Na^+ current were recorded at room temperature with the whole-cell patch-clamp technique (Hamill *et al.*, 1981). Outward current refers to the flow of cations from the pipette (cytosolic side) to the bath (external side). The pipette solution contained (in mM): 100 KGluconate, 40 KCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 4 EGTA/KOH (10 nM free Ca^{2+}), 2 K_2ATP, 10 HEPES (pH 7.2). The bath solution contained (in mM): 140 NaCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES (pH 7.2). A concentration of 10 μM amiloride was used to measure the amiloride-induced hyperpolarization or the amiloride-induced current change.

RNA preparation and Northern blot analyses

The fetal alveolar cells were washed twice with ice-cold phosphate-buffered saline, scraped into 0.3 ml lysis solution/35 mm diameter dish [4 M guanidinium thiocyanate, 20 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 0.7% β-mercaptoethanol] and frozen immediately. Total RNA was extracted with the acidic phenol method (Chomczynski and Sacchi, 1987). Northern blot analysis was carried out using standard techniques (Sambrook *et al.*, 1989). Briefly, denatured RNAs (5 μg/lane) were separated on 1% agarose gels in 1 × MOPS (pH 5.8), 2.2 M formaldehyde and transferred to nylon membranes (Hybond-N, Amersham). The membranes were hybridized in 5 × SSC, 30% formamide, 0.1% SDS, 5 × Denhardt's solution and 100 μg/ml denatured herring sperm DNA with a restriction fragment (*Eco*RI–*Kpn*I 614 bp fragment) of the entire rat colon Na^+ channel cDNA (Lingueglia *et al.*, 1993), labelled by random priming with [α-^{32}P]dCTP. After washing at 65°C in 2 × SSC–0.1% SDS, the membranes were exposed from 6 h to 2 days (Kodak XOMAT AR film). All the experiments were carried out at least twice on more than three different cultures.

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